BLOOD CELL FORMATION AND DISTRIBUTION IN RELATION TO THE MECHANISM OF THYROID-ACCELERATED METAMORPHOSIS IN THE LARVAL FROG.

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PLATES 34 TO 36.

A study of blood formation in the adult frog has shown that the spleen is the chief hemopoietic organ (Jordan and Speidel, 1923). Red bone marrow may appear for a brief period in the spring, following the hibernation period, and become a locus of hemopoietic activity as pointed out by Jordan (1919) for the leopard frog, but this condition does not persist for more than a few weeks. For the remainder of the year the spleen is practically the sole organ of blood formation. In the larval frog, however, the kidney plays an important part in this process. The intertubular regions of the tadpole kidney are packed with many erythrocytes and leucocytes, together with many transitional forms. The spleen is small and of relatively minor importance. Apparently the kidney is the chief hemopoietic organ of the tadpole. At some time in the later developmental history of the frog, therefore, the locus of hemopoiesis becomes shifted from the kidney to the spleen. This is a clear case of what may be termed successive hemopoietic dimorphism.

It occurred to the writers that it might be of interest to find out whether the hemopoietic locus could be rapidly shifted experimentally by treating tadpoles with thyroid extract and thus inducing metamorphosis. The experiments performed to test this point have also thrown some light upon the part played by blood cells in metamorphic differentiation. In spite of the large number of investigations within recent years in the field of amphibian metamorphosis, no study has been made of the part played in the process by blood cell formation

and distribution. The importance of such a study is apparent when consideration is given to the fact that metamorphosis is fundamentally related to a change in metabolic rate, and must, therefore, involve hemopoiesis. Our experiments also indicate some features of the thyroid mechanism, some links in the complicated chain of reactions initiated by the thyroid secretion, by which its profound effects are accomplished in various regions of the body. Finally, the results corroborate previous conclusions of the writers in regard to the functions of the lymphocyte.

Material and Methods.

Bullfrog tadpoles (*Rana catesbeiana*) were used for the experiments. The first batch, fourteen in number, was collected early in January near the University of Virginia, and the second batch, twenty-two in number, on February 20th. These were judged to be about 9 or 10 months old. They averaged from 6 to 7 cm. in length, and possessed small insignificant hind legs.

Metamorphosis was initiated by feeding thyroid extract of sheep. A dry tablet of the extract was softened with water and small fragments inserted into the mouth of the tadpole on the tip of forceps. Control specimens were kept in a separate aquarium and killed at intervals throughout the course of the experiments. The tissues were in all cases fixed in the formalin-Zenker mixture of Helly and stained by the eosin-azure method of Giemsa.

The results given in this paper are based chiefly upon these thirty-six animals. In addition, however, observations have been made on fifty-three other tadpoles of greatly varying sizes collected during the months of March, April, and May.

EXPERIMENTS AND OBSERVATIONS.

In the first series of experiments metamorphosis was initiated in nine tadpoles. These either died or were killed, at intervals varying from 4 to 14 days after the first thyroid treatment. Normal controls were also examined. In the second series of experiments metamorphosis was initiated in ten tadpoles. These also either died or were killed, at intervals varying from 2 to 14 days after the first thyroid administration. Normal controls were also killed. Microscopic study was made of the tissues of these animals, including kidney, spleen, intestine, mesentery, fore and hind limbs, axillary lymph nodule, liver, thymus, head region, skin, and tail.

Attempts were also made to bring about complete metamorphosis. With one exception, these were unsuccessful, although some of the

animals lived for more than a month. Metamorphosis proceeded rapidly at first, but after about 10 days or 2 weeks usually slowed up, and the specimens died in the resulting more or less quiescent condition of "metamorphic stasis."

External Changes.

The early changes in the tadpole following thyroid administration are well known. Externally the most conspicuous changes are tremendous increase in vascularity and rapid growth of the hind limbs, shrinkage in body size due principally to the extrusion of the intestinal contents and to the shortening of the intestine, absorption of the tail, appearance of the fore limbs (the left fore limb always appearing first), and the gradual change from the tadpole type of face to the frog type.

Internal Changes.

A. The Kidney.—The kidney becomes smaller and somewhat paler. Upon section it is found to contain fewer blood cells. In the normal tadpole kidney the intertubular regions contain many blood cells, including both adult types of erythrocytes and leucocytes and those in their developmental stages (Fig. 1). After thyroid treatment, however, the intertubular regions become partially drained of their blood cells (Fig. 2). Although there is less hemopoietic tissue present in the kidney after thyroid treatment, it is nevertheless quite active. Many lymphocytes are in process of amitotic division, and in addition some mitotic figures may be seen. Lymphocytes, special granulocytes (neutrophils), eosinophils, together with transitional stages between lymphocytes and the eosinophilic and special granulocytes, are quite numerous. Transitional stages between lymphoid hemoblasts and erythrocytes may also be found. In no case is there total drainage or exhaustion of the hemopoietic capacity of the kidney.

B. The Spleen.—The spleen is somewhat variable in its response to thyroid treatment. In some animals in which metamorphosis proceeded rather slowly, the spleen was somewhat larger than normal, and its hemopoietic activity slightly increased. There is some evidence that mesenchymal cells in the nearby mesentery differentiate into lymphocytes, migrate to the spleen and become incorporated in it, thus contributing to its growth. In most of the animals, however, the spleen does not increase in size. It remains approximately the normal size, or becomes smaller, with its erythropoietic activity greatly heightened.

The chief function of the normal tadpole spleen is the production of erythrocytes from lymphoid hemoblasts. These, together with the cells of the splenic reticulum, are the predominating types of cells (Fig. 3). Thyroid administration markedly stimulates the differentiation of erythrocytes from lymphocytes. 2 days after the first feeding the spleen is characterized by the presence of large numbers of young erythrocytes, together with many intermediate stages between

these and their lymphocyte progenitors. In later stages the erythrocytes are carried off by the blood vessels, so that the percentage of lymphocytes to erythrocytes in the spleen increases. In those animals that reach a condition of metamorphic stasis, the spleen becomes partially or totally exhausted of its hemopoietic content (Fig. 4). Both lymphocytes and erythrocytes are drained from the spleen to be taken into the general circulation. These cells are not replaced rapidly enough to meet the demand set up by the increased metabolic rate induced by the thyroid treatment.

C. *The Tail and Intestine* (*Regions of Regressive Change*).—Following thyroid treatment there is an increase in the leucocyte content of the tail and of the intestine. The predominating types of leucocytes in the tail region are the special granulocytes, lymphoid phagocytes (phagocytes derived from lymphocytes), and to a less extent, lymphocytes and eosinophils. The special granulocytes contain no particles of cellular debris and perform little, if any, true phagocytosis. Tissues undergoing degeneration are removed by the lymphoid phagocytes. The special granulocytes may go to pieces and then be themselves ingested by the phagocytes. Apparently the occasional eosinophils in the tail region have a like history. The lymphocytes in the tail are possibly about to differentiate into phagocytes.

The intestine of a thyroid-treated tadpole is characterized by the presence in the mucosa of many eosinophils and lymphoid phagocytes. Some quiescent lymphocytes are also in evidence. There is plainly a tremendous loss of cellular elements by process of phagocytosis and by process of sloughing off into the intestinal lumen. This latter point has been recently stressed by Kuntz (1922). Eosinophils do not perform phagocytosis here, but in many cases appear to go to pieces in the lining of the intestine and are then ingested by the phagocytes. The complete absence of special granulocytes is striking.

D. *The Limbs* (*Regions of Progressive Change*).—Very conspicuous is the increased vascularization of the limbs a few days after the first thyroid feeding. Outside of the blood vessels the predominating type of leucocyte is the lymphocyte, and it is, furthermore, practically the only type of blood cell present here. In the normal tadpole there is a region of mesenchymal cells beneath the epidermis (Fig. 5). After thyroid treatment, however, a rather definite and continuous layer of lymphocytes appears here (Fig. 6). Some of these lymphocytes may come from the general circulation but the appearances suggest rather that they are mostly differentiated *in situ* from the mesenchymal cells. Occasional transition stages between mesenchymal cells and lymphocytes can be seen.

E. *Other Regions.*—The skin of the ventral, lateral, and dorsal trunk region, of a thyroid-treated animal which showed this lymphocyte layer in the rapidly growing limbs, was found to contain no similar subepidermal layer of differentiating lymphocytes. Examination was also made of the thymus, liver, minute axillary lymph nodule, and the head region, but the findings are not pertinent to our present interests and may be omitted in this paper.

INTERPRETATION AND DISCUSSION.

Thyroid administration raises the metabolic rate of the larval frog. This brings about profound changes in the larval hemopoietic organization and in the blood cell distribution. Our findings indicate that (1) an increased demand is set up for erythrocytes to furnish the physical basis for the maintenance of the new high oxidative level. Erythropoiesis is, therefore, stimulated in both spleen and kidney, especially in the former. (2) Increased metabolic rate involves also a demand for leucocytes, (a) for granulocytes and phagocytes to aid in the processes of regressive change, (b) for lymphoid hemoblasts which are the progenitors of erythrocytes as well as of granulocytes, (c) for lymphocytes to aid in stimulating growth of adjacent tissues in regions of progressive change. These matters will now be discussed more fully.

The demand for blood cells of all kinds is followed by partial drainage of erythrocytes and leucocytes from the kidney and spleen, and by the stimulation of both of these organs to increased hemopoietic activity. The kidney now produces chiefly leucocytes, the spleen erythrocytes. At this new high metabolic rate the spleen becomes a much more important organ. If the hemopoietic reserve is not drained too rapidly by excessive thyroid feeding, the spleen may be slowly stimulated to an increase in size in spite of the demands being made upon it. In animals treated in this way it may be said that partial drainage of the kidney, together with stimulation of the spleen, indicates that the shift of hemopoietic locus from the kidney to the spleen has been initiated. In no case so far have we been able to carry the shift to completion experimentally although some of our animals have been fed thyroid for more than a month.

Most of our thyroid-treated animals have died from 10 to 30 days after the first feeding. The initial rapid metamorphic differentiation progressively becomes slower until a condition of metamorphic stasis is reached, death usually occurring in this stage. The fundamental cause of death we believe to be anemia. The spleen of a tadpole in true metamorphic stasis is practically exhausted of its hemopoietic capacity. The erythrocytes are first drawn from the spleen, then the lymphoid hemoblasts are stimulated to differentiate into erythrocytes, and the latter are then in turn taken up by the general circula-

tion.   Lymphocytes as such may also be drawn from the spleen.   The splenic reticular cells may round up to differentiate into lymphocytes, and other lymphocytes may be brought into the spleen from other parts of the body.   In any event, if the demand for blood cells is too great, the spleen is ultimately exhausted of its hemopoietic content and metamorphic stasis results, associated with a condition of anemia. Upon the success of the organism in meeting the tremendous hemopoietic demands of thyroid-accelerated metamorphosis, depends whether or not metamorphosis will be successfully completed.   This conclusion does not preclude the possibility that metamorphosis may also be inhibited by other factors, such as the lack of proper coordination between the thyroid and the hypophysis.

In an earlier series of experiments upon adult frogs we have induced a marked increase in the hemopoietic rate by means of experimental hemorrhage, by repeated aspiration of blood directly from the heart (Jordan and Speidel, 1923).   The results have shown that the ability of the frog to withstand successfully such treatment depends upon the ability of the lymphocytes, as progenitors of both leucocytes and erythrocytes, to form, to proliferate, and to differentiate rapidly enough into blood cells to meet the demands of the experiment.   A similar result was obtained with the injection of saponin, a hemolytic toxin. Spleens totally or partially exhausted of their hemopoietic content were obtained in these cases.   In the light of these experiments upon adult frogs, the writers were able to predict, before making any microscopic examination, that the spleen of a tadpole which had died in metamorphic stasis would show exhaustion of its hemopoietic content.

It is of interest in this connection to note that Swingle (1923) has attempted to bring tadpoles through this stage of metamorphic stasis by treatment with both thyroid and hypophysis transplants. He finds the response variable.   In a few of the animals he believes the treatment is efficacious, in others not.   It is difficult for the writers to see how the addition of the active principle of the hypophysis could help an anemic tadpole over the condition of metamorphic stasis unless it stimulated the production of more blood cells, a function which has never been ascribed to the hypophysis.   It is probable that the tadpoles which were helped over this condition still possessed some hemopoietic

reserve. The writers do not mean to ignore the fact that metamorphosis is an extremely complicated process, the successful completion of which requires proper interaction between thyroid and hypophysis, and possibly other tissues. For the interaction to bring about the completed metamorphosis, however, it is also necessary that there be an adequate hemopoietic basis.

The phagocytic action of leucocytes in certain regions during metamorphosis is well known. Large mononuclear phagocytes derived from lymphocytes do most of the actual work of ingestion and removal of disintegrating cellular debris in both tail and intestine. In the intestinal mucosa are found many eosinophils, mostly gathered near the basement membrane of the epithelial lining. Our study of these cells suggests that they give off substances, often themselves going to pieces in the process, that aid in causing disintegration or other regressive changes of nearby tissues. They perform no phagocytosis, but may themselves be taken up later and removed by the lymphoid phagocytes. Kuntz (1922) points out that much of the intestinal lining is finally removed by being extruded into the lumen and passed out with the feces. The granulocytes in the tail region also appear to play no important part in the actual ingestion and phagocytosis of degenerating cells, but rather aid in breaking down surrounding tissues, thus rendering them more easy of ingestion and phagocytosis by the phagocytes. In brief, the granulocytes, both special and eosinophilic, appear to be mobile, unicelluar glands which give off substances that have a lytic effect on tissues undergoing regressive change. The writers do not hold that the secretions of these cells are the first cause of degeneration in these regions. As Morse (1918) has pointed out, a spontaneous breaking down of tissues occurs before mobilization of the granulocytes. Whatever initiates the degeneration process, however, the granulocytes aid in furthering it.

That certain leucocytes have in addition a growth-promoting function in certain regions of the tadpole during metamorphosis is also probable. Carrel (1922) found in the case of the chick that either leucocyte extract, or fluid in which leucocytes have been living for some time, when added to embryonic heart or fibroblast tissue cultivated *in vitro*, stimulated 100 per cent greater growth and differentiation than occurred in the control cultures. He concludes that leuco-

cytes, whether living or dead, give off substances which stimulate adjacent resting cells to grow and differentiate.   We believe that the rapid growth of the limbs in thyroid-treated tadpoles may be partly ascribed to the stimulating effect of lymphocytes.   Carrel's experiments dealt with all leucocytes taken together, a mixed culture.   In the involuting tadpole there is a specific differential distribution of leucocytes, the lymphocyte being easily the predominant type in the limbs (Fig. 6).   It is obvious, therefore, that any leucocytic secretion in this region must come from the lymphocytes.   We suggest, accordingly, that the growth-promoting function of leucocytes, as demonstrated by Carrel, is due principally, if not entirely, to the lymphocyte component of leucocytes.   Again, the writers do not hold that the lymphocytic secretion is necessarily the first cause of the growth effect in the limbs. But whatever initiates the growth reaction, the lymphocytes apparently aid in maintaining it.   From the negative standpoint, it is hardly probable that either the granulocytes or the phagocytes, which are found in abundance in regions of regressive change, also give off growth-promoting substances.

This conception that the lymphocyte, in addition to its other functions, is a mobile unicellular gland with the function of stimulating growth of nearby cells need not be a surprising one.   The lymphocyte is an embryonic type of cell, relatively undifferentiated.   As such it is an element with pluripotential capacities.   It is a well known fact that adult resting cells, when cultivated in vitro, are stimulated to grow when embryonic juice is added to the culture.   The lymphocyte may be regarded as an embryonic relic that persists throughout life, a cell that is on hand at all times to bring stimulation (to contribute "embryonic juice") to any region where stimulation is needed.

The criticism may be made that, since the tadpole is an embryo, the limbs contain a supply of embryonic juice derived from the muscles, mesenchyme, etc., which should furnish the stimulation for growth. The answer to this is that the tadpole limbs, in the stages used in our experiments, are in a comparatively inactive condition and remain so for a considerable period of time.   The larval bullfrog takes from 2 to 3 years to arrive at the period of metamorphosis.   Histologically, the structure of the skin and connective tissue of the hind limb suggests a rather stable condition, approximating an adult type.   These tissues, therefore, resemble adult tissues rather than embryonic.
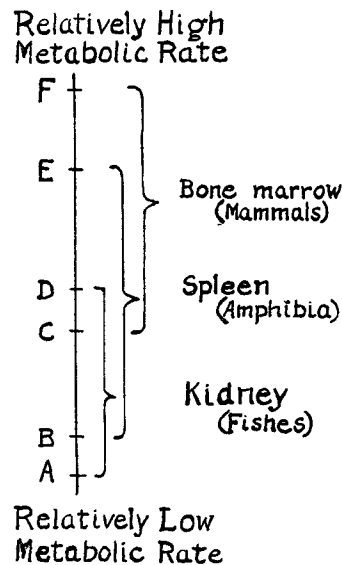
The conception of the lymphocyte as a growth-promoting cell leads to a consideration of lymphoid tissue in relation to growth. Scammon (1921) has constructed a curve showing the age variation in growth and size of lymphoid organs. These attain their greatest size and weight in man at about the age of puberty. It is interesting to note that this is also the period of considerable stimulation to new growth of many parts of the body.

In both embryonic and adult developmental changes it is known that successive "waves of growth" occur in the various organs and parts of an animal. Just as the lymphocytes aid in growth promotion of the limb tissues of a tadpole in metamorphosis, so they may be a part of the mechanism for bringing about and maintaining the less conspicuous increase in growth rate that occurs in vertebrates at definite periods in various organs and parts of the body.

Our findings are readily harmonized with Huxley's (1922, 1923) general conception of the mechanism of metamorphosis. The initiation of the shift of hemopoietic locus from the kidney to the spleen represents merely one aspect of the change from larval organization to adult organization, like the change from gills to lungs, or from the fin and tail method of locomotion to that by means of limbs. The larval hemopoietic organ, the kidney, is chiefly a leucocyte producer and functions best at the lower metabolic level. With the onset of metamorphosis its cells are largely drained and not adequately replaced. The adult hemopoietic organ, the spleen, is chiefly an erythrocyte producer and functions best at the higher metabolic rate. It is important to note, too, that at the higher metabolic rate there is a greater stimulation of erythropoiesis, a condition which probably explains why the spleen becomes the chief adult organ of hemopoiesis.

In the warm-blooded vertebrates the spleen is superseded as erythrocyte producer, this function being taken over by the red bone marrow. In these forms the metabolic rate is much higher than in the amphibia, and it is concluded that the bone marrow is here better adapted for erythrocyte production at the higher metabolic rate than is the spleen. In agreement with this idea is the observation that red bone marrow appears in the frog (*Rana pipiens*) in the early spring, after the hibernation period, at the time when there is great demand for new blood to support the rapidly increasing metabolic rate associated with the

breeding process.   The life history of the frog, therefore, with respect
to hemopoietic centers, presents a case of successive hemopoietic tri-
morphism.   This is correlated to some extent, at least, with changes
in the rate of metabolism.   As shown in the diagram (Text-fig. 1),
the kidney functions best at relatively low metabolic levels (A to D),
the spleen at medium levels (B to E), and the bone marrow at high levels
(C to F).   Kidney and spleen may function together at a certain

Relatively High
Metabolic Rate

F

E   Bone marrow
                        (Mammals)

D   Spleen
C   (Amphibia)

                        Kidney
B   (Fishes)
A

Relatively Low
Metabolic Rate

TEXT-FIG. 1. Diagram to show the relation between a successive trimorphism
of hemopoietic centers and rate of metabolism. A to D, range of metabolic rate
at which the kidney functions as a hemopoietic center. B to E, range of metabolic
rate at which the spleen functions as a hemopoietic center. C to F, range of meta-
bolic rate at which the bone marrow functions as a hemopoietic center.

range of metabolic levels, as B to E.   This condition occurs in the tad-
pole.   Spleen and red bone marrow may be active at the same time at
the range of metabolic levels, C to E, a condition that may be found in
the leopard frog for a brief period in the spring.   Or even all three
organs may be active at a certain level, C to D.   This last condition
was found in an adult bullfrog examined in the spring.   The spleen
was most active, the bone marrow next, and the kidney least.   A

similar condition was found in one of our thyroid-treated tadpoles, an animal which completed its metamorphosis and died 33 days after the first feeding.

Our comparative studies indicate that there is also a parallel phylogenetic trimorphism of hemopoietic centers as we pass up the vertebrate series. The chief hemopoietic organs in teleost fishes are kidney and spleen; in anuran amphibia, kidney, spleen, and transient red bone marrow; in birds and mammals, red bone marrow, lymph nodes, and spleen. The order seems to be, (1) kidney, (2) spleen, (3) red bone marrow. Correlated with this seriation in a general way, perhaps, is the fact that the thyroid, a regulator of metabolic rate, is of relatively small size in fishes, of medium size in amphibia, and largest in birds and mammals.

SUMMARY.

1. Thyroid-accelerated metamorphosis in the larval frog is accompanied by changes in the hemopoietic centers and in the blood cell distribution in the various regions of the body. These changes are interpreted as results of the fundamental change in basal metabolic rate induced by the thyroid treatment.

2. There is initiation of the shift of hemopoietic locus from the kidney, the larval hemopoietic organ, to the spleen, the adult hemopoietic organ. The spleen, being chiefly an erythrocyte producer, becomes of greater importance with the transition from the lower metabolic rate to the higher, since greater erythropoiesis becomes necessary to supply the physical basis for the maintenance of the higher metabolic rate.

3. It is suggested that the appearance of red bone marrow in the later history of the frog is correlated with a still higher metabolic rate. Phylogenetically, in the vertebrate series, red bone marrow is also associated with higher metabolic rate.

4. The new metabolic rate initiated in tadpoles by thyroid administration sets up a demand for (a) erythrocytes, (b) granulocytes and lymphoid phagocytes for distribution to regions of regressive change, (c) lymphocytes, (1) as progenitors of erythrocytes, granulocytes and phagocytes, (2) for promoting growth of cells in regions of progressive change.

5. Upon the hemopoietic reserve, which in the last analysis is the lymphocyte (and its mesenchymal precursor), depends the extent to which metamorphosis will proceed. Inability on the part of the hemopoietic centers, chiefly the spleen, to keep pace with the demand for blood cells during metamorphosis results in metamorphic stasis, a condition of anemia which is usually followed by death.

6. The growth-promoting function of leucocytes, as demonstrated by Carrel, is probably to be ascribed to the lymphocyte component of leucocytes.

7. The granulocytes have probably also a glandular function, and may exert a lytic effect upon adjacent tissues in regions of regressive change.

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EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Longitudinal section of kidney of normal bullfrog tadpole. Parts of three tubules may be seen. The intertubular region is filled with many erythrocytes, leucocytes, and transitional forms. × about 500.

FIG. 2. Longitudinal section of kidney of thyroid-treated tadpole, killed 4 days after the first feeding. The intertubular regions are largely drained of blood cells. (This area somewhat exaggerates the average amount of drainage.) × about 500.

PLATE 35.

FIG. 3. Cross-section of spleen of normal tadpole. The principal cellular constituents are erythrocytes, lymphocytes, and splenic reticular cells. × 1,000.

FIG. 4. Cross-section of spleen of a thyroid-treated tadpole that died in metamorphic stasis. The spleen is almost totally drained of its hemopoietic content. × 1,000.

PLATE 36.

FIG. 5. Vertical section of skin of hind limb of a normal tadpole showing epidermis, e, and dermis, d. In the dermis may be seen mesenchymal and connective tissue cells and fibers. Note the stable, definitive, histological appearance of this skin. × 1,000.

FIG. 6. Vertical section of skin of hind limb of a thyroid-treated tadpole in which rapid growth is taking place. In the dermis, d, just beneath the epidermis, e, may be seen a conspicuous layer of lymphocytes, l. × 1,000.
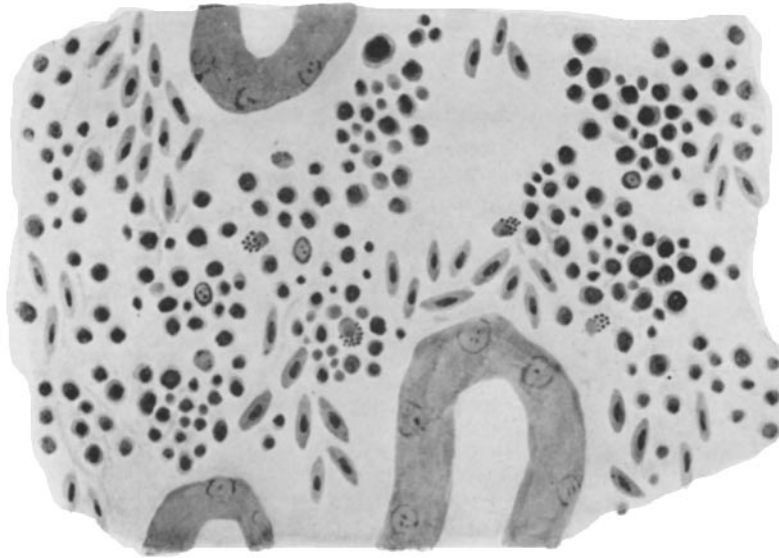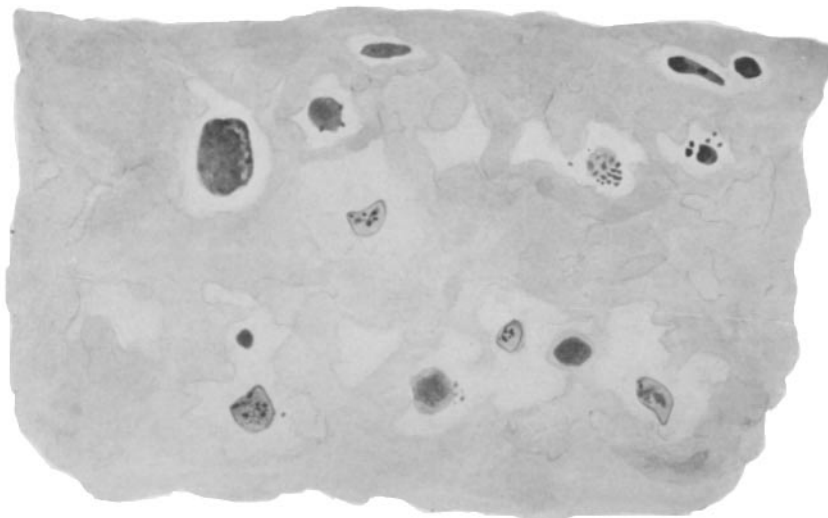
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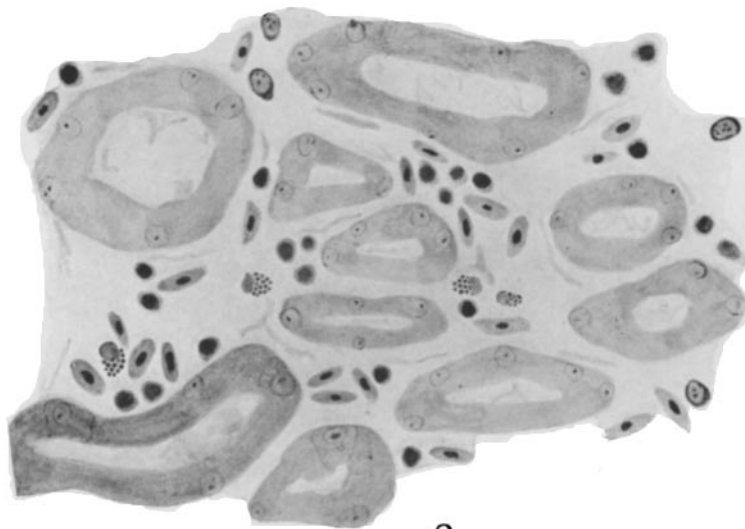
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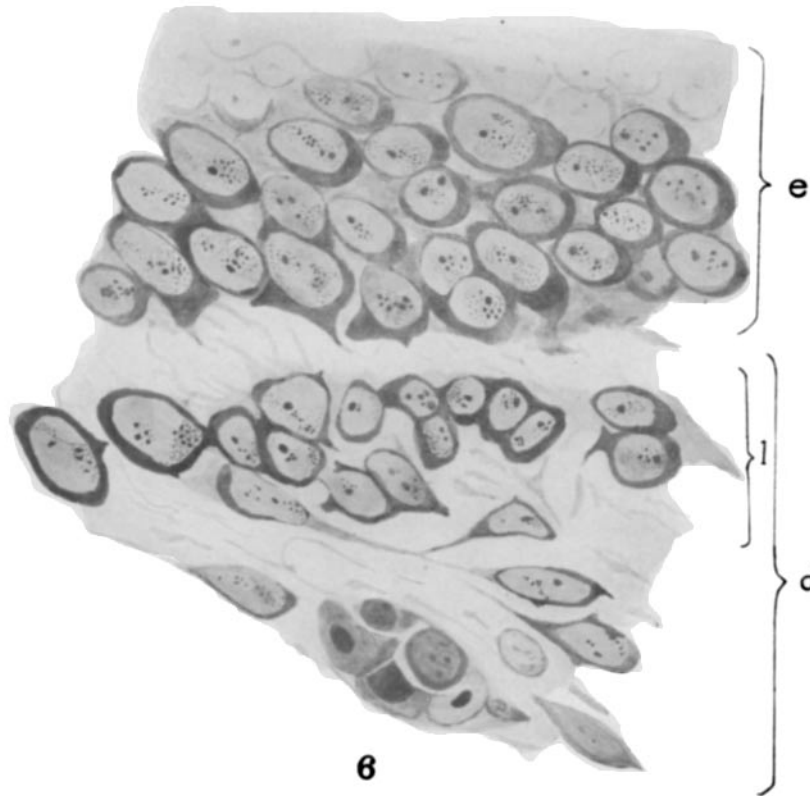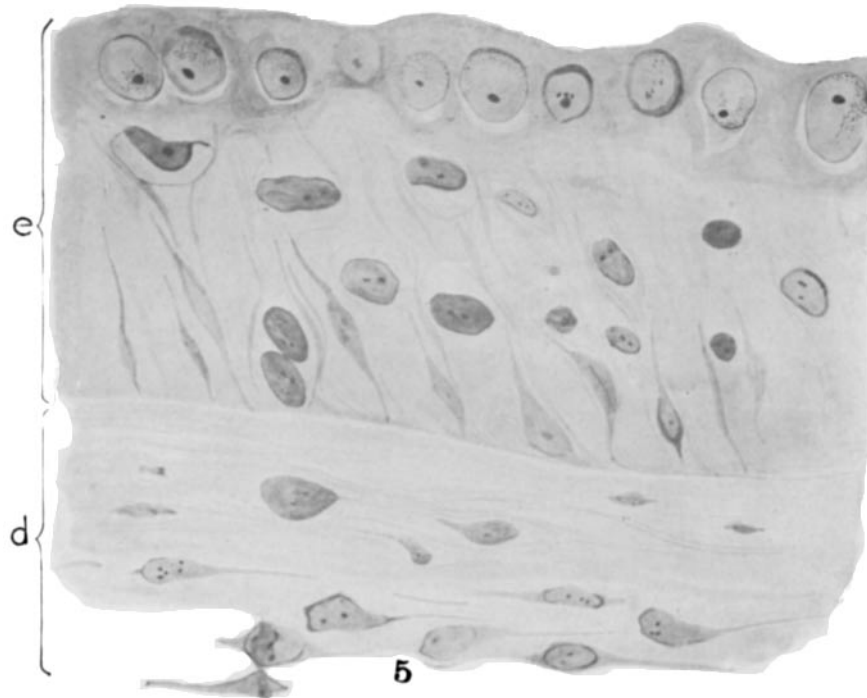(Jordan and Speidel: Blood cell formation and distribution.)

3

4

(Jordan and Speidel: Blood cell formation and distribution.)

(Jordan and Speidel: Blood cell formation and distribution.)